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In Vitro Activities of Eight Antifungal Drugs against 104 Environmental and Clinical Isolates of *Aureobasidium pullulans*

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***Aureobasidium pullulans* is an unusual agent of phaeohyphomycosis. The *in vitro* activities of antifungals against 104 isolates of *Aureobasidium pullulans* var. *pullulans* and *A. pullulans* var. *melanigenum* revealed low MIC_{90}s of amphotericin B, posaconazole, and itraconazole. However, they were resistant to fluconazole (≥64 μg/ml) and had high MICs of voriconazole, isavuconazole, caspofungin, and micafungin.**

Aureobasidium pullulans is a common ubiquitously distributed melanized yeast-like fungus that is an occasional agent of phaeohyphomycosis (1). The genus *Aureobasidium* phylogenetically belongs to *Ascomycota*, order *Dothideales*, family *Dothideaceae* (2, 3). The clinically significant species are *A. pullulans* (4) and *A. proteae* (5); a case of *Aureobasidium mansoni* associated with cerebral phaeohyphomycosis (6) probably concerned a misidentification. The fungus commonly occurs in the hospital environment, where it colonizes moist surfaces, such as glass and metal, and has recently been reported to cause a varied spectrum of infections, including peritonitis, meningitis, and fungemia (1).

Although *Aureobasidium pullulans* has been reported to be the most common species in over 32 cases of human phaeohyphomycosis infections, so far there are no systematic data on its antifungal susceptibility profiles. Furthermore, the fungus has not been identified to the species level in the majority of cases reported so far. Herein we report antifungal susceptibility profiles of 104 molecularly characterized isolates of *Aureobasidium pullulans*. The isolates were obtained from the reference centers Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands, and the University of Texas Health Science Center, San Antonio, TX, USA. The strains included currently recognized varieties with their type strains: *Aureobasidium pullulans* var. *pullulans* ($n = 51$) and *A. pullulans* var. *melanigenum* ($n = 53$). Most strains originated from clinical ($n = 51$) and environmental ($n = 47$) samples, while 4 strains were from unknown sources (see Table S1 in the supplemental material). Most *A. pullulans* var. *melanigenum* strains (43 of 51) were recovered from clinical specimens, whereas *Aureobasidium pullulans* var. *pullulans* strains (40 of 51) were mainly isolated from the environment. Of the clinical *A. pullulans* var. *melanigenum*, 38 isolates (74.5%) were reported from cases of systemic infections in the United States. Species and variety identities were confirmed by sequencing the internal transcribed spacer region and the D1/D2 region of the nuclear large subunit rRNA gene (7).

Antifungal susceptibility testing was performed in duplicate as per CLSI document M38-A2, with some modifications (8). Briefly, isolates were cultured on potato dextrose agar in the dark (25°C) for up to 7 days to induce sporulation. Inocula were prepared by scraping the surface of the fungal colonies with a cotton swab moistened with sterile physiological saline containing 0.05% Tween 40. Large particles were allowed to settle for 5 min, and then a suspension of spores was adjusted with a spectrophotometer (Spectronic 20D; Milton Roy, Rochester, NY) to 68 to 71% transmission (at 530 nm) and diluted 5-fold to yield a final inoculum of 1.5×10^4 to 5×10^4 CFU/ml as controlled by quantitative colony counts. Reagent-grade powders of amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central, Sandwich, United Kingdom), posaconazole (Merck, Whitehouse Station, NJ), and isavuconazole (Basilea Pharmaceuticals, Basel, Switzerland) were used in a concentration range of 0.016 to 16 μg/ml. The fluconazole (Pfizer) concentration ranged from 0.063 to 64 μg/ml, while the caspofungin (Merck) and micafungin (Astellas Pharma, Ibaraki, Japan) concentrations ranged from 0.008 to 8 μg/ml. The MIC/minimum effective concentration (MEC) distributions of *A. pullulans* var. *pullulans* and *A. pullulans* var. *melanigenum* were compared by using the Mann-Whitney-Wilcoxon test for skewed distribution. A P value of <0.05 was considered statistically significant.

Results were read for 95% of isolates after an incubation of 72 h at 25°C and for the remaining isolates, which did not grow luxuriantly, after 96 h. MICs of amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole were defined visually as the lowest drug concentration that prevented any discernible growth (100% inhibition), whereas for fluconazole, the MIC was taken as the lowest concentration supporting ≥50% growth inhibition compared to the growth in the control wells. For caspofungin and micafungin, MECs were determined microscopically

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TABLE 1 Geometric mean MICs, MIC ranges, MIC_{50}, and MIC_{90} of antimycotic agents against *A. pullulans* var. *pullulans* and *A. pullulans* var. *melanigenum*

Fungus (no. of strains) and drug	MIC (μg/ml)[a]			
	Range	50%	90%	Geometric mean
Total isolates (104)				
Amphotericin B	≤0.016–16	0.5	1	0.32
Fluconazole	4–≥64	64	≥64	52.75
Itraconazole	≤0.016–16	0.25	0.5	0.17
Voriconazole	≤0.016–16	1	2	0.77
Posaconazole	≤0.016–4	0.25	0.5	0.12
Isavuconazole	0.016–16	2	4	1.29
Caspofungin	0.063–8	1	4	1.17
Micafungin	≤0.008–8	0.25	2	0.28
A. pullulans var. *pullulans* (51)				
Amphotericin B	0.031–1	0,5	1	0.35
Fluconazole	8–≥64	≥64	≥64	52.19
Itraconazole	≤0.016–2	0.063	0.5	0.08
Voriconazole	0.125–16	1	4	0.79
Posaconazole	≤0.016–1	0.031	0.25	0.05
Isavuconazole	0.016–8	1	2	0.63
Caspofungin	0.063–8	1	8	1.31
Micafungin	≤0.008–8	0.125	1	0.15
A. pullulans var. *melanigenum* (53)				
Amphotericin B	≤0.016–16	0.25	1	0.3
Fluconazole	4–≥64	64	≥64	53.29
Itraconazole	≤0.016–16	0.25	1	0.35
Voriconazole	≤0.016–8	1	2	0.76
Posaconazole	≤0.016–4	0.25	0,5	0.25
Isavuconazole	0.016–16	4	4	2.59
Caspofungin	0.25–4	1	2	1.05
Micafungin	0.016–4	0.5	2	0.5

[a] 50% and 95%, MIC_{50} and MIC_{90}, respectively.

as the lowest concentration of drug promoting the growth of small, round, compact hyphae relative to the appearance of the filamentous forms seen in the control wells. Quality control strains of *Paecilomyces variotii* (ATCC 22319), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258) were included in each assay.

The MIC_{50}, MIC_{90}, and geometric mean (GM) values *viz. Aureobasidium pullulans* var. *pullulans* and *A. pullulans* var. *melanigenum* are given in Table 1. All isolates had low MICs of amphotericin B (MIC_{90}, ≤1 μg/ml), itraconazole (MIC_{90}, 0.5 μg/ml), and posaconazole (MIC_{90}, 0.5 μg/ml). However, the less active drugs were fluconazole, voriconazole, isavuconazole, micafungin, and caspofungin. The highest GM MICs were 52.75 μg/ml for fluconazole, followed by 1.29 μg/ml for isavuconazole and 1.17 μg/ml for caspofungin. In contrast much lower GM MICs were obtained for voriconazole (0.77 μg/ml), amphotericin B (0.32 μg/ml), micafungin (0.28 μg/ml), itraconazole (0.17 μg/ml), and posaconazole (0.12 μg/ml). Anidulafungin was not tested but is expected to be comparable to micafungin. Routine testing of caspofungin for *Candida* has been cautioned recently because of large interlaboratory variability (9). During the analyses of this large collection of *Aureobasidium* isolates, we did not observe more than one dilution difference of caspofungin tested in duplicate. We evaluated whether the MIC/MEC values correlated with the two varieties using Mann-Whitney-Wilcoxon test for skewed distribution. A statistically significant difference in susceptibilities between the two varieties was observed for itraconazole, posaconazole, isavuconazole, and micafungin ($P < 0.001$) but not for amphotericin B, fluconazole, voriconazole, and caspofungin ($P \geq 0.05$). No differences were recorded between clinical and environmental isolates.

Most of the clinical isolates tested in this study belonged to *A. pullulans* var. *melanigenum*, which were found to be susceptible to posaconazole, itraconazole, and amphotericin B. Amphotericin B has been used successfully to treat *A. pullulans* infections in cases of systemic dissemination, meningitis, peritonitis (10), and two proven cases of fungemia (11, 12). Previously, antifungal susceptibility testing (AFST) of solitary isolates of this fungus reported in two cases indicated amphotericin B MICs of 0.25 μg/ml and 0.5 μg/ml (10, 12, 13). Recently published guidelines on treatment of phaeohyphomycosis (14) also recommend therapy with amphotericin B in cases due to *A. pullulans* infections. In this study, *A. pullulans* isolates were resistant to fluconazole. While there are no established interpretive criteria for antifungal susceptibilities of *A. pullulans*, the high MICs of all isolates tested suggest fluconazole resistance. Previously, two cases in which *A. pullulans* was the etiologic agent reported both high (64 μg/ml) and low (4 μg/ml) MICs of fluconazole (10, 13). Amphotericin B, itraconazole, and posaconazole were the drugs with the best overall activity. This is the first comprehensive study on antifungal susceptibility data of a large number of clinically significant *A. pullulans* species demonstrating that the two different varieties of *A. pullulans* show low MICs of amphotericin B, itraconazole, and posaconazole, which supports the therapeutic recommendations published by the European Society of Clinical Microbiology and Infectious Diseases/European Confederation of Medical Mycology (ESCMID/ECMM) (14).

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